2013

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Christopher J. Schell
University of Chicago, cschell@uchicago.edu

Julie K. Young
Utah State University, julie.k.young@aphis.usda.gov

Elizabeth V. Lonsdorf
Utah State University, elizabeth.lonsdorf@fandm.edu

Rachel M. Santymire
Franklin and Marshall College, rsantymire@lpzoo.org

Journal of Mammalogy, 94(5):1131–1140, 2013

Anthropogenic and physiologically induced stress responses in captive coyotes

Christopher J. Schell,* Julie K. Young, Elizabeth V. Lonsdorf, and Rachel M. Santymire

Committee on Evolutionary Biology, University of Chicago, 900 E 57th St. CULV 402, Chicago, IL 60637, USA (CJS)
United States Department of Agriculture–Wildlife Service–National Wildlife Research Center, Predator Research Facility, and Department of Wildland Resources, Utah State University, USDA-WS-NWRC Predator Research Facility, Department of Wildland Resources, USU - BNR 163, Logan, UT 84322-5292, USA (JKY)
Department of Psychology, Franklin and Marshall College, Lancaster, PA 17603, USA (EVL)
Conservation and Science, Lincoln Park Zoo, 2001 N. Clark St., Chicago, IL 60614, USA (RMS)

* *Correspondent: cschell@uchicago.edu*

Repeated stressful events can negatively impact overall health by continuous stimulation of the hypothalamic–pituitary–adrenal axis, which leads to depletion of glucose stores and suppression of immune and reproductive function. The influence of stressors on survivability is particularly salient for coyote (*Canis latrans*) populations, because understanding how coyotes cope with stressors may provide relevant context on coyote adaptation to urbanized ecosystems. Our objectives were to physiologically validate fecal glucocorticoid metabolite (FGM) analysis in coyotes by performing an adrenocorticotropic hormone (ACTH) challenge in 12 captive individuals (6 treatment and 6 control) housed at the United States Department of Agriculture National Wildlife Research Center Predator Research Facility in Millville, Utah; to quantify potential changes in FGM output due to diurnal variation and sex; and to determine the effects of 2 anthropogenic events (placement of a novel cooling fan and state holiday celebrations) on the coyotes' stress responses (via FGM production). Results demonstrated that in response to the ACTH injection, treatment animals (3 males and 3 females) displayed FGM concentration peaks $\geq$ 5-fold (range: 5- to 30-fold) above their preinjection means approximately 8 h after injection, which was a greater ($P = 0.037$) response than control animals. FGM output was lowest for morning fecal samples compared with midday ($P = 0.001$) and evening ($P < 0.001$) samples. Within the evening period, FGM output for male samples tended to be higher ($P = 0.056$) than for female samples, although not significant. The anthropogenic events elicited FGM concentration peaks $\geq$ 5-fold above pre-event means for several of the study animals occurring approximately 12 and 9 h later, respectively. This study is the 1st to physiologically validate the measurement of stress physiology using FGM analysis in coyotes and demonstrate the impact of anthropogenic events on their stress response. Furthermore, this work provides a foundation for future studies of FGMs, stress, and anthropogenic effects in wild and captive systems.

Key words: adrenocorticotropic hormone (ACTH) challenge, anthropogenic stressors, *Canis latrans*, diurnal variation, enzyme immunoassay, fecal glucocorticoid metabolite analysis, stress response

DOI: 10.1644/13-MAMM-A-001.1

Coyotes (*Canis latrans*) provide an interesting system to examine stress physiology because of the species' ability to successfully persist in nonnative environments. Recent ecological literature has suggested that increased fragmentation, development, and reduction of tertiary predators such as gray wolves (*Canis lupus*) in multiple niches facilitated coyote geographic expansion (Grubbs and Krausman 2001a; Fox 2006). Coyotes are relatively flexible in their landscape use, enabling them to survive despite environmental change (Bekoff and Wells 1980, 1986; Séquin et al. 2003; Gehrt 2007). Changes to coyote home-range size and movement patterns (Way et al. 2001; Tigas et al. 2002; Riley et al. 2003; Gehrt et al. 2009), habitat use and diet (Grinder and Krausman 2001a; Randa and Yunger 2006; Morey et al. 2007; Grubbs and Krausman 2009), health and disease (Grinder and Krausman 2001b; Liccioli et al. 2012), and social interactions (Atwood et

al. 2004) have been documented in multiple urban and suburban landscapes. However, no study has evaluated potential changes to stress physiology as a result of increased urban living or validated methods to analyze glucocorticoids in this species. The instances of stochastic events, novel stimuli, and chronic stressors are arguably greater in metropolitan areas compared with native environments, and increased chronic stress negatively influences overall health and survival (Love and Williams 2008; Schulkin 2011). Despite these factors, coyotes in metropolitan areas do not have greater disease prevalence compared with other populations, and population densities are increasing (Grinder and Krausman 2001b). Therefore, measuring glucocorticoids may be an informative tool in understanding how coyotes cope with nonnative environments.

Monitoring adrenocortical activity also may prove valuable in determining the physiological influence social group dynamics have on coyotes. Unlike related Canidae social systems (e.g., African wild dogs [*Lycaon pictus*] and gray wolves, coyote populations may persist in an array of resident and transient individuals across a landscape (Atwood et al. 2004; Fox 2006; Gehrt 2007). Territorial incursions by transients or neighboring residents into home ranges of resident coyotes can result in conflict (Gese et al. 1996; Gese 1998, 2001). Additionally, competition for and reinforcement of dominance status within a group can result in instances of physical (e.g., biting and chasing) and behavioral (e.g., teeth baring and scent marking—Gese et al. 1996; Gese 1998, 2001) aggression. These instances of conflict may differentially increase and affect adrenocortical activity of an individual as a function of social status, subsequently influencing overall health. Consequently, glucocorticoid analysis may prove beneficial in examining the effects of social stimuli on coyote adrenocortical activity.

The biological stress response is characterized by the activation of the hypothalamic–pituitary–adrenal axis, which is a negative feedback system that regulates the production of glucocorticoids (Jacobson and Sapolsky 1991; Herman et al. 2003; Ulrich-Lai and Herman 2009). At the onset of hypothalamic–pituitary–adrenal—axis activation, corticotropin-releasing hormone is released by the hypothalamus and stimulates the anterior pituitary gland, resulting in the production of adrenocorticotropic hormone (ACTH). The ACTH then stimulates the adrenal cortex to release glucocorticoids, such as cortisol and corticosterone (Dedovic et al. 2009). These glucocorticoids circulate (via blood) back to the hypothalamus as part of the negative feedback loop to cease hypothalamic–pituitary–adrenal—axis stimulation (Dedovic et al. 2009; Schulkin 2011). Short-term activation of the hypothalamic–pituitary–adrenal axis is highly adaptive, because the production of glucocorticoids mobilizes energy stores via increased gluconeogenesis, suppresses secondary physiological functions (e.g., immune, reproductive, etc.), and attenuates memory retention. Conversely, overproduction of glucocorticoids in the long term depletes available glucose, resulting in several physiological issues that can decrease overall health and fecundity (Love and Williams 2008; Schulkin 2011). As a result, chronic stress can be a significant threat to the fitness of an organism by disrupting proper hypothalamic–pituitary–adrenal—axis function.

Noninvasive fecal glucocorticoid metabolite (FGM) monitoring is ideal for wildlife species for several reasons. First, repeated capture events and blood draws of wild individuals are impractical and can result in rapid increases in serum glucocorticoid concentrations due to handling stress that ultimately influence subsequent analysis of adrenocortical activity (Möstl and Palme 2002; Touma and Palme 2005; Santymire et al. 2012). Second, plasma glucocorticoids only sample a short time period and are more susceptible to fluctuations due to handling or blood draws (Harper and Austad 2000; Möstl and Palme 2002; Santymire and Armstrong 2010; Santymire et al. 2012). In contrast, fecal glucocorticoid metabolites provide an inclusive view of adrenocortical activity over a 12- to 48-h period and are less vulnerable to rapid fluctuations in glucocorticoid concentrations (Wielebnowski et al. 2002; Loeding et al. 2011; Santymire et al. 2012). The validation and analysis of FGMs has been used on several canid species including domestic dogs (*Canis lupus familiaris*—Schatz and Palme 2001), gray wolves (Sands and Creel 2004), red wolves (*C. lupus rufus*—Young et al. 2004), African wild dogs (Monfort et al. 1998), and maned wolves (*Chrysocyon brachyurus*—Vasconcellos et al. 2011).

Our goal was to develop noninvasive methods to study the influence of stressors on overall health, which is particularly salient for coyote populations, because understanding how coyotes cope with stressors may provide relevant context on coyote adaptation to urbanized ecosystems. Our objectives were to physiologically validate FGM analysis in coyotes by performing an ACTH challenge in 12 captive individuals (6 treatment and 6 control) housed at the United States Department of Agriculture National Wildlife Research Center (NWRC) Predator Research Facility in Millville, Utah; to quantify potential changes in FGM output related to diurnal variation and sex; and to determine the effects of anthropogenic events (placement of a novel cooling fan and state holiday celebrations) on the coyotes' stress responses (via FGM production). We hypothesized that individuals receiving the ACTH injection would demonstrate peak FGM concentrations greater than those receiving saline injections and that the FGM concentrations would be greater for females compared with males. Previous literature on Carnivora has demonstrated higher glucocorticoids in females compared with males (African wild dogs [Creel et al. 1997], domestic dogs [Schatz and Palme 2001], and cheetahs [*Acinonyx jubatus*—Wielebnowski et al. 2002]), although this trend is not demonstrated consistently in the clade (African wild dogs [Monfort et al. 1998], and gray wolves [Sands and Creel 2004]). Additionally, we predicted that FGM output would gradually increase from morning to evening samples because coyotes are mainly crepuscular (Way et al. 2001; Gehrt 2007); thus, we anticipated higher FGMs in the evening and midday due to the lag time between circulating plasma hormones and excreted fecal

Table 1.—Descriptive values detailing the treatment condition, proximity to the facility entrance, total injection dose, fold-increase values for the adrenocorticotropic hormone (ACTH) injection and anthropogenic stressors (cooling fan introduction and Pioneer Day festivities), and excretion lag times for the ACTH injection and anthropogenic stressors for each study animal. Excretion lag times are reported as total hours and minutes elapsed between experience of a stressor and excretion of fecal glucocorticoid metabolite (FGM) peak values. FGMs were observed from 17 July 2011 to 1 August 2011 at the National Wildlife Research Center (NWRC) Predator Research Facility in Millville, Utah. F = female, M = male, NA = not available.

					ACTH		Cooling fan		Pioneer Day	
Identification	Sex	Treatment group	Proximity to facility entrance (m)	Total ACTH given (IU)	Fold increase	Excretion lag time	Fold increase	Excretion lag time	Fold increase	Excretion lag time
06102	F	ACTH[a]	4	40.4	19	7 h 33 min	1	24 h 01 min	3	9 h 46 min
1032	F	ACTH[a]	8	42.2	5	7 h 33 min	4	9 h 57 min	2	9 h 29 min
1052	F	ACTH[a]	12	41	30	7 h 29 min	2	24 h 00 min	1	23 h 40 min
0422	F	Control	2	46.6	17	7 h 37 min	16	9 h 58 min	6	9 h 40 min
06052	F	Control	6	39.6	7	7 h 36 min	3	24 h 00 min	0	9 h 40 min
1050	F	Control	10	41.8	2	17 h 35 min	0	24 h 00 min	5	9 h 33 min
06063	M	ACTH[a]	3	47	13	7 h 32 min	9	9 h 58 min	9	9 h 24 min
1051	M	ACTH[a]	7	46.2	12	10 h 53 min	2	9 h 53 min	10	9 h 30 min
1071	M	ACTH[a]	11	46.2	13	7 h 28 min	2	9 h 48 min	1	9 h 40 min
08171	M	Control	1	45.6	4	7 h 24 min	7	9 h 58 min	18	9 h 40 min
06133	M	Control	5	42.2	4	7 h 45 min	5	9 h 55 min	1	23 h 41 min
1041	M	Control	9	46.2	3	7 h 31 min	0	24 h 00 min	NA	NA

[a] The ACTH dosage was 4 IU/kg.

hormonal metabolites. Finally, we predicted that salient anthropogenic stressors would reliably correlate with FGM peaks witnessed post–anthropogenic event. We conducted this study in captivity specifically to be able to monitor and account for prominent environmental or anthropogenic stressors, in addition to obtaining repeated fecal samples from known individuals.

Materials and Methods

Animals.—Twelve captive-born individuals (6 males and 6 females) ranging in age from 1.0 to 7.0 years (3.0 ± 0.7 years [$\bar{X} \pm SEM$]) were housed at the NWRC. Coyotes at the facility were housed in multiple enclosure types ranging from large outdoor pens (0.1–6.0 ha) to kennel environments (3.3 m^2). Study animals were moved to outdoor raised kennels (3.3 m^2) on 5 July 2011 and given 12 days to acclimate to their environment prior to fecal sample collection. To reduce the stress response to researcher presence during collection events, we approached animals daily at 0800, 1300, and 1800 h starting on 13 July 2011, which corresponded to the collection schedule. Animals were fed 650 g of commercial mink food (Fur Breeders Agricultural Cooperative, Logan, Utah) daily and water was provided ad libitum (Brummer et al. 2010). Additionally, kennels were cleaned daily in accordance with standard operating procedures for the Millville site (SOP: AC/UT 001.00 Daily coyote check and care for Millville Predator Research Facility).

Adrenocorticotropic hormone challenge.—We randomly selected coyotes for 1 of 2 treatment groups: control (saline) and treatment (ACTH). Both groups had an equal number of males and females ($n = 6$; 3 males and 3 females). The ACTH (Sigma-Aldrich, St. Louis, Missouri) dosage was 4 IU/kg given intravenously, determined by a prior challenge experiment conducted in 2011 (S. French, Utah State University, pers. comm.). The ACTH (range: 40.4–47 IU total) and saline (range: 39.6–46.6 IU total) injections were administered from 0757 to 0831 h on 27 July 2011 (Table 1). All animals were weighed a week before injection to determine proper doses. Immediately before all injections, doses were drawn up in saline solution and animals were moved into standard NWRC den boxes to physically restrain them during injection. This capture procedure is standard at the NWRC facility and is used to reduce injury to the animals and staff.

The time of injection was alternated by sex and treatment, with the exception of 2 anxious individuals (1 control female and 1 control male), which were injected 1st to reduce the potential for injury. Average amount of time to restrain, inject, and release each animal was 0 h 2 min ± *SD* 1 min (range: 1–4 min). After the injection period, animals were kept in the den boxes and all kennels were cleaned to ensure fresh fecal samples were collected. Individuals were then released back into their kennels, as staff and researchers withdrew from the area to reduce potentially stressful activity. Total time to inject all animals was 38 min, and total time of human presence on site was 127 min from initial arrival to the end of kennel cleaning.

We collected fecal samples from 17 July to 1 August 2011, 2 or 3 times daily at 0800–0900 h (AM period), 1300–1400 h (midday: MD period), and 1800–1900 h (PM period). Additionally, all samples defecated were collected 5 days post–ACTH injection to ensure that samples containing the ACTH-induced peaks would be observed in our analyses. Time of collection was recorded and multiple samples collected within the same time frame were ordered by freshness. We determined sample freshness by location of the defecated sample compared with samples collected previously during the

day or week, in addition to stiffness and overall appearance. During collection events, several samples were found in pools of urine. These samples were considered contaminated and were not collected for analysis. All samples were immediately stored at –20°C to limit the amount of hormone metabolite degradation. Samples were then shipped overnight on dry ice to the Lincoln Park Zoo Endocrinology Laboratory (Chicago, Illinois) for FGM analysis. All research conforms to guidelines of the American Society of Mammalogists for research on live animals (Sikes et al. 2011), and was approved through the University of Chicago Institutional Animal Care and Use Committee (ACUP 72185), Lincoln Park Zoo Research Committee, and the NWRC Institutional Animal Care and Use Committee (QA-1809).

Diurnal variation and sex differences in FGM output.—Samples collected during the study period also were used to examine potential diurnal variation and sex differences in FGM output. Briefly, samples were collected from 17 July to 1 August 2011, 2 or 3 times daily. Additionally, samples were collected at different periods during the day (AM, MD, and PM) to compare FGMs across collection periods. All animals were sampled during the same time period each day. Samples that were contaminated by urine were not collected.

Previous literature has demonstrated that degradation of glucocorticoid hormones due to bacterial decomposition occurs near linearly over a 12-h period, which could influence diurnal variation in FGM output (Möstl and Palme 2002; Shutt et al. 2012). This study does not specifically examine differences in FGM output due to increasing ambient environmental exposure. However, to account for this issue we cleaned and monitored kennels daily to obtain fecal samples as close to defecation as possible. Further, captive coyotes at the NWRC facility defecate 3 or 4 times daily, with the majority of fecal samples defecated from 0500 to 0900 h (staff, NWRC, pers. obs.).

Anthropogenic stressors.—An anthropogenic stress event was characterized as a human-associated event resulting in a loud audible noise or visual stimuli that occurred within or near the kennel environment. Several minor events were documented during the study period; however, 2 major events also occurred. On the morning of 21 July 2011, a large cooling fan was introduced into the kennel area near the facility entrance. A few days later marked the beginning of Pioneer Day, which is a Utah state holiday celebrated with a combination of fireworks and parades. During the 2011 year, approximately 25,000 people participated in the day's activities from the morning of 23 July and continued into 24 July 2011. Neighboring residents near the NWRC facility used fireworks on 22 July 2011, and continued use throughout the weekend. One control male did not defecate from the morning of 23 July to 26 July 2011, and was therefore excluded from Pioneer Day analyses. The date and diurnal period (AM, MD, or PM) were noted when the events occurred.

Fecal sample processing.—All samples were freeze-dried on a lyophilizer (Thermo Modulyo Freeze Dryer; Thermo Scientific, Waltham, Massachusetts) for 3 days and crushed to a fine powder before extraction using previously described methods (Brown et al. 1994; Santymire et al. 2012). Briefly, samples were then weighed (0.2 ± *SD* 0.02 g), combined with 5.0 ml of 90% ethanol (ethanol:distilled water), and agitated on a mixer (Glas-col, Terre Haute, Indiana) for 30 min (setting 60). The samples were then centrifuged for 20 min at 1,500 rpm at 10°C, and the supernatant was poured into clean glass tubes. The fecal pellet was resuspended in 5.0 ml of 90% ethanol, vortexed for 30 s, and recentrifuged for 15 min at 1,500 rpm. The supernatant was poured into the corresponding glass tubes and the combined supernatants were dried down under air and a hot-water bath (60°C). Once dry, all samples were reconstituted with 2.0 ml of phosphate-buffered saline (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, NaCl), vortexed briefly, and sonicated for 20 min before analysis.

Enzyme immunoassay.—We examined the effectiveness of 2 previously described corticosterone and cortisol enzyme immunoassays (Young et al. 2001; Santymire and Armstrong 2010; Heintz et al. 2011; Santymire et al. 2012) to observe coyote FGMs. Polyclonal cortisol antiserum (R4866), corticosterone antiserum (CJM006), and horseradish peroxidase were provided by C. Munro (University of California, Davis, California). Cortisol antiserum, corticosterone antiserum, cortisol horseradish peroxidase, and corticosterone horseradish peroxidase were used at dilutions of 1:8,500, 1:15,000, 1:20,000, and 1:15,000, respectively (Santymire and Armstrong 2010; Heintz et al. 2011). The enzyme immunoassays were biochemically validated by demonstrating parallelism between binding inhibition curves of fecal extract dilutions (1:2–1:1,024), the cortisol standard ($R^2 = 0.980$), and the corticosterone standard ($R^2 = 0.976$); and significant percent recovery (> 90%—Santymire and Armstrong 2010; Santymire et al. 2012) of exogenous cortisol (1:1,500; $\hat{y} = 0.8702x + 8.2525$, $R^2 = 0.9955$) and corticosterone (1:600; $\hat{y} = 0.848x + 22.642$, $R^2 = 0.9385$) added to pooled fecal extracts. Assay sensitivity was 1.95 pg/well and intra- and interassay coefficients of variation were < 10% for both enzyme immunoassays. Further, we used Pearson product moment correlation to compare our cortisol and corticosterone standards with serially diluted fecal extracts (Santymire et al. 2012). Our cortisol enzyme immunoassay had a higher correlation ($r = 0.997$) compared with our corticosterone enzyme immunoassay ($r = 0.968$). As a result, we used cortisol as our primary enzyme immunoassay for this study.

Data analyses.—We tested FGM data for normality using the Shapiro–Wilk test statistic. Data that were not normally distributed were natural log–transformed. For physiological validation, samples collected 72 h preinjection ($n = 3$ or 4) for each individual were averaged and compared to elevated values post–ACTH injection for each individual. We used fold increase to quantify the FGM response to the ACTH injection (Monfort et al. 1998; Brown et al. 1999). Specifically, fold increase was determined by computing the quotient between the pre–ACTH injection mean and the FGM peaks (Table 1). To determine if fold-increase values differed

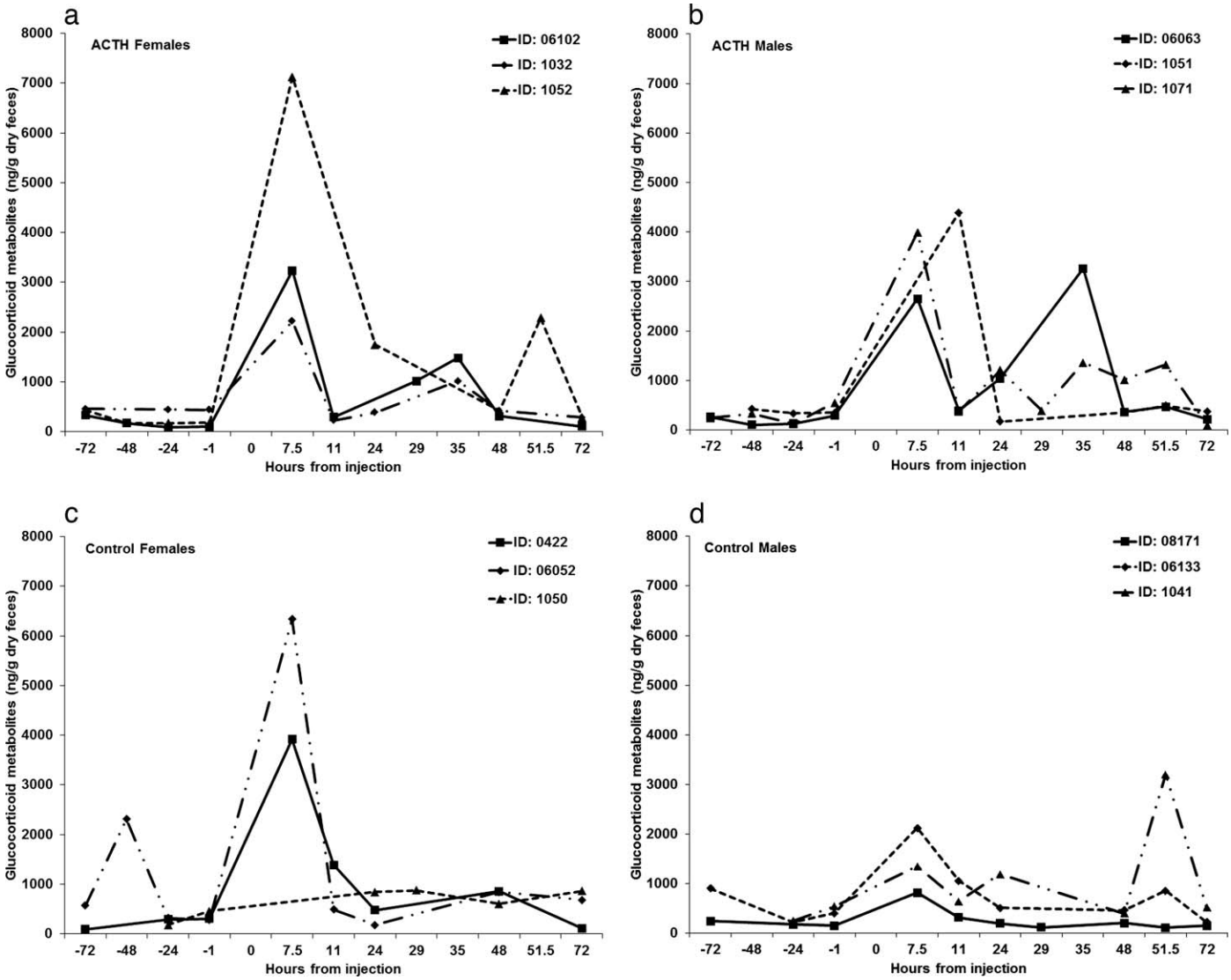

Fig. 1.—Fecal glucocorticoid metabolite (FGM) concentrations for a) females given adrenocorticotropic hormone (ACTH; $n = 3$), b) control females ($n = 3$), c) males given ACTH ($n = 3$), and d) control males ($n = 3$) 72 h before and after injections. The ACTH (range: 40.4–47.0 IU total) and saline (range: 39.6–46.6 IU total) injections were administered from 0757 to 0831 h on 27 July 2011 (hour 0) at the National Wildlife Research Center (NWRC) Predator Research Facility in Millville, Utah.

as a function of treatment condition or sex, we utilized Mann–Whitney *U*-tests for post–ACTH injection data. Additionally, we used Spearman rank correlation analyses to determine if order of injection, mass, or proximity to the facility entrance was related to ACTH fold-increase values. Time of ACTH injection was compared to time of collections postinjection to determine the approximate excretion lag time for FGMs.

To determine the potential for diurnal variation (comparing AM, MD, and PM samples) we used mixed regression models to measure the influence of time on FGMs. We specified collection period as the main fixed effect and animal identification as a random effect to account for repeated measures from the same individual. Sex, treatment condition, and animal mass also were specified as fixed effects in the model. The model was fitted with a random intercept and slope to examine whether individual differences in FGMs were consistent within subjects. Additionally, paired *t*-tests were performed to further examine diurnal variation of FGM output within subjects. Results were Bonferroni adjusted to account for multiple comparisons between collection periods (AM, MD, and PM). We excluded peak FGM values 7.5 h (11 h for coyote 1051) post–ACTH injection from these analyses.

Fold increase in response to anthropogenic stressors was quantified similarly to our ACTH results. Briefly, fold increase in response to anthropogenic events was quantified by computing the quotient between the pre-event means and the FGM peaks; then Mann–Whitney *U*-tests were used to determine differences as a function of a priori treatment condition and sex. Spearman rank correlation analyses were used to determine if animal mass or proximity to the facility entrance had any relationship with fold increase in FGMs post–anthropogenic event. Time of the anthropogenic stressor was

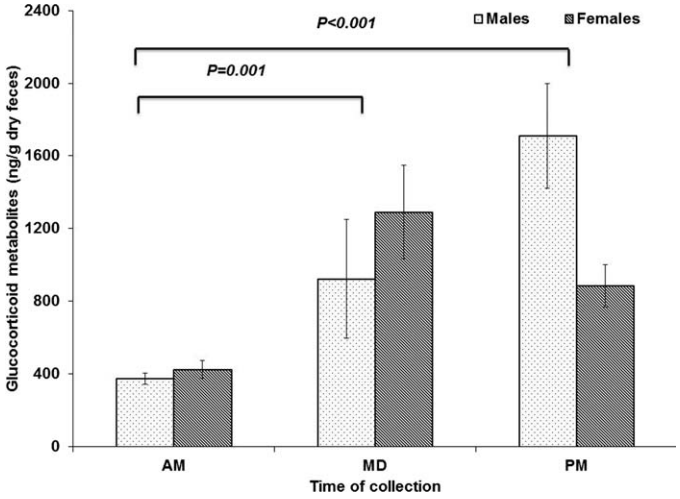

FIG. 2.—Mean (± *SE*) fecal glucocorticoid metabolites (FGMs) over time (0800–0900 h [AM], 1300–1400 h [midday: MD], and 1800–1900 h [PM] for all study coyotes. Peak FGM values 7.5 h (11 h for coyote 1051) post–adrenocorticotropic hormone injection were excluded from these analyses.

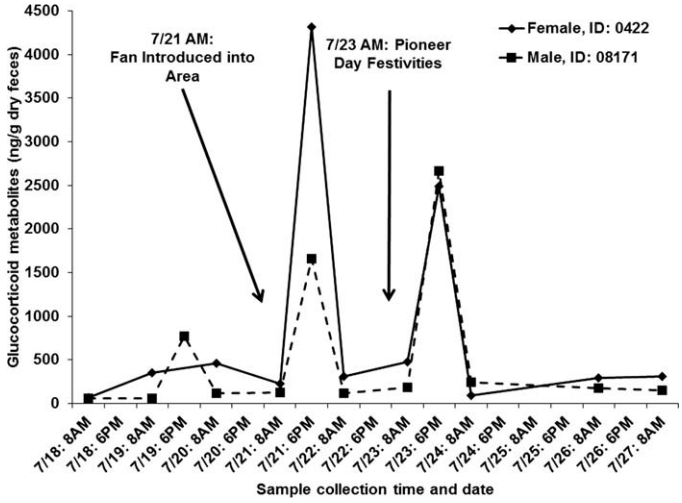

FIG. 3.—Fecal glucocorticoid metabolite (FGM) concentrations of 2 study coyotes (1 male and 1 female) representative of the sample population that experienced pronounced FGM peaks to the observed environmental events.

compared to time of collection postevent to determine the approximate excretion lag time. These anthropogenic excretion lag-time data were then compared to our ACTH challenge for further biological validation. All statistical analyses were performed using SYSTAT 11 (Systat Software, Inc. 2008). Results are presented as mean ± *SE*, where *P*-values < 0.05 were considered significant and *P*-values between 0.051 and 0.1 were considered a trend. Cortisol metabolite data were used for all analyses.

RESULTS

Adrenocorticotropic hormone challenge.—Pre–ACTH injection averages did not differ by treatment ($n = 12$, $t_{10} = -1.155$, $P = 0.275$), sex ($n = 12$, $t_{10} = 0.440$, $P = 0.670$), or mass ($b = -0.449$, $t_{10} = -1.587$, $P = 0.144$). Fold-increase values above the pre–ACTH injection mean were greater (Mann–Whitney $U_{11} = 31.00$, $P = 0.037$) in the treatment group (5- to 30-fold) compared with the control group (1- to 17-fold). Peak values postinjection for all ACTH animals were at least 5-fold higher than the preinjection average; 3 control individuals (2 females and 1 male) had comparable fold-increase values (Table 1). There was no relationship between fold-increase values and order of injection ($\rho = -0.014$, $P = 0.964$), or fold-increase values and kennel proximity to the facility entrance ($\rho = 0.0$, $P = 1.0$). Approximate excretion lag time for all individuals was 8 h 39 min ± 0 h 10 min (range: 0724–1735 h; 0 h 10 min, Fig. 1; Table 1).

Diurnal variation in FGM output.—Overall FGM output did exhibit diurnal variation ($z = -9.055$, $SE = 0.078$, $P < 0.001$). Post hoc paired *t*-tests demonstrated significant differences between AM and MD FGMs ($t_{10} = -4.416$, $P = 0.001$), and AM and evening FGMs ($t_{11} = -6.481$, $P < 0.001$). MD and PM FGM values were not statistically different ($t_{10} = -0.994$, $P = 0.343$; Fig. 2). There was a trend for greater FGMs in males ($t_{10} = -2.163$, $P = 0.056$; Fig. 2) compared with females for the PM samples. Additionally, individual variation in FGM increase from AM to PM samples was consistent within individuals (random effect intercept: $z = 3.441$, $SE = 2.292$, $P = 0.001$). Sex ($z = -0.440$, $SE = 0.139$, $P = 0.660$), treatment condition ($z = -0.322$, $SE = 0.102$, $P = 0.748$), and mass ($z = -0.657$, $SE = 0.209$, $P = 0.511$), however, did not influence the diurnal variation observed.

Anthropogenic effects on FGM concentrations.—In response to the cooling fan introduced to the kennels, 4 coyotes (3 males and 1 female) produced ≥ 5-fold increases in FGM output, similar to the ACTH challenge. The approximate excretion lag time for the cooling fan introduction (11 h 58 min ± 5 h 53 min; range: 9 h 55 min–24 h; Table 1; Fig. 3) also was similar to the ACTH challenge excretion lag time. There was a significant negative relationship ($\rho = -0.720$, $P = 0.006$) between fold-increase values and proximity to the facility entrance, with closer individuals more likely to witness fold increases similar to the ACTH challenge (Table 1). There were no differences a priori between assigned ACTH and control animals (Mann–Whitney $U_{11} = 16.00$, $P = 0.749$) or between males and females (Mann–Whitney $U_{11} = 15.00$, $P = 0.631$; Table 1).

In response to Pioneer Day festivities, 5 coyotes (3 males and 2 females) produced ≥ 5-fold increases in FGM output with an approximate excretion rate of 9 h 21 min ± 5 min (range: 9 h 24 min–9 h 46 min; Table 1; Fig. 3). Similar to the fan introduction, there was a significant negative relationship ($\rho = -0.609$, $P = 0.027$) between fold-increase values postevent and proximity to the facility entrance, with closer individuals more likely to witness fold increases similar to the ACTH challenge (Table 1). Again, there were no a priori differences between assigned treatment groups (Mann–Whit-

ney $U_{10} = 14.00$, $P = 0.855$) or between males and females (Mann–Whitney $U_{10} = 8.00$, $P = 0.201$; Table 1).

Discussion

This study was the 1st to physiologically validate the measurement of FGM via an ACTH challenge to noninvasively assess adrenocortical activity in coyotes. All treatment individuals had an ACTH-induced peak approximately 8 h after the injection. This excretion lag time is rapid compared to results from related Canidae. Lag time between ACTH injection and peak glucocorticoid values for African wild dogs (Monfort et al. 1998), gray wolves (Sands and Creel 2004), and maned wolves (Vasconcellos et al. 2011) are ~24, 16–20, and ~20 h, respectively. This difference may partly be due to various sizes within the Canidae: the coyote is currently the smallest species of the family for which adrenocortical activity has been physiologically validated. However, this trend is not extended to all of Carnivora, because smaller species in the order have longer lag times between ACTH injection and peak glucocorticoid concentrations (20–44 h in black-footed ferrets [*Mustela nigripes*—Young et al. 2001]). Other carnivores demonstrate longer excretion lag times (15 h for grizzly bears [*Ursus arctos horribilis*—Hunt and Wasser 2003], 25 h for brown hyenas [*Hyaena brunnea*—Hulsman et al. 2011], and 16 h for spotted hyenas [*Crocuta crocuta*—Benhaiem et al. 2012]). It is more likely that the method of injection influenced the rapid excretion rate, because all other previous ACTH studies within Canidae administered the injection intramuscularly rather than intravenously (Monfort et al. 1998; Sands and Creel 2004; Young et al. 2004; Vasconcellos et al. 2011). Our intravenous injection would have resulted in the direct transfer of ACTH to the bloodstream (Möstl and Palme 2002; Touma and Palme 2005). It is important to note that the reduced excretion lag time also may be partially explained by the frequent (3 or 4 times daily) defecation events of the captive coyotes.

Fold increases above the preinjection mean were significantly greater for treatment group individuals compared to control individuals. The ACTH males and females experienced increased FGM output 12- to 13-fold and 5- to 30-fold above their preinjection means, respectively. Monfort et al. (1998) demonstrated similar pronounced responses to ACTH administration in African wild dogs. Fecal corticosteroid metabolites in male and female African wild dogs increased approximately 9- and 19-fold above pre-ACTH concentrations (Monfort et al. 1998). The similar fold increase in African wild dogs is surprising given that the study animals from Monfort et al. (1998) were given 400 IU compared with a maximum injection of 47 IU for this study. Results from Monfort et al. (1998) are similar to those of Young et al. (2004), who utilized a 140 IU dose for red wolves and observed only a 4- to 11-fold increase in FGMs above pre-ACTH concentrations. In contrast, Sands and Creel (2004) administered a lower dosage of 0.5 IU/kg of ACTH to gray wolves and the 2 treatment gray wolves demonstrated a 5-fold increase above the pre-ACTH concentrations.

This variation in FGM fold increase among Canidae may suggest that coyotes are more sensitive to hypothalamic–pituitary–adrenal—axis stimulation. More broadly, an increased attenuation to glucocorticoids could influence physiological adaptation of coyotes to repeated stressors. However, differences in methodologies across studies may partially explain the variation in fold-increase values. First, contrary to our study, Monfort et al. (1998) and Sands and Creel (2004) used a corticosterone and cortisol radioimmunoassay, respectively. Young et al. (2004) used both a corticosterone radioimmunoassay and a cortisol enzyme immunoassay for ACTH analyses. Second, the other authors used varying methodologies to extract glucocorticoids from the feces; and the specific ACTH compound used differed across studies. Third, potential differences in species' metabolism may account for the variance in fold-increase values. As a result, it is difficult to directly compare fold-increase values across these studies.

It is important to note that FGM concentrations for 3 control individuals did emulate fold increases of the treatment group, with FGM peaks $\geq$ 4-fold above the preinjection means. These results are likely due to the actual stress experienced during handling and injection. Previous literature on multiple wildlife species has demonstrated FGM increases (Harper and Austad 2000) and plasma glucocorticoid increases (Morton et al. 1995; Kenagy and Place 2000) due to handling and capture. Examination of data from Harper and Austad (2000) showed increased FGM responses due to brief handling during captive cage transfer in 3 species of Rodentia (house mice [*Mus musculus*], deer mice [*Peromyscus maniculatus*], and red-back voles [*Myodes gapperi*]). Similarly, results from Kenagy and Place (2000) on wild-caught female yellow-pine chipmunks (*Tamias amoenus*) demonstrated plasma glucocorticoid increases in response to trap capture for 1–3 h. Our results further suggest that capture, restraint, and handling stress can augment FGM concentrations. Previous ACTH challenge papers on related Canidae (domestic dog [Schatz and Palme 2001], gray wolf [Sands and Creel 2004], red wolf [Young et al. 2004], African wild dog [Monfort et al. 1998], and maned wolf [Vasconcellos et al. 2011]) did not have established control groups to compare with ACTH animals, making it difficult to know whether those study animals also responded to capture and restraint similar to our control individuals.

Interestingly, examination of our data demonstrates diurnal variation in FGM concentrations. Specifically, FGM output was greatest in MD and PM samples compared with AM samples. This pattern may be occurring for several reasons. First, this trend may reflect the circadian pattern of a crepuscular species due to the 8- to 12-h delay of metabolism (from blood to feces). According to Touma and Palme (2005) it might be almost impossible to detect circadian patterns in carnivores primarily because of an infrequent diet or longer digestive tract. However, it is possible that the constant diet of our captive coyotes may have inadvertently augmented their

diurnal fluctuations in glucocorticoids. Second, staff activity around the kennel area is greatest in the morning and early afternoon (0730–1600 h), and dissipates in the evening hours (1600–2400 h). It is likely that increased staff presence influences circulating glucocorticoids early in the day, and those influences are reflected in the feces at MD and PM collection periods. Third, the diurnal variation observed may be an artifact of reduced FGM concentrations from increased bacterial enzymatic activity in morning samples (Möstl and Palme 2002; Shutt et al. 2012). Morning samples potentially had longer exposure time to ambient temperatures; the elapsed time between PM and AM collection periods (13 h) is greater than between AM and MD (4 h) or MD and PM (4 h) periods. Shutt et al. (2012) previously described a near-linear decrease in FGMs over a 12-h period when feces had prolonged exposure to ambient conditions. However, given that our captive coyotes often defecated from 0500 to 0900 h, the potential for increased environmental exposure of AM samples is low. Further research that controls for degradation potential is needed to properly address the effects of feeding and human activity on circadian and seasonal patterns in FGMs.

Contrary to our initial prediction, our study did not demonstrate sex differences between preinjection means or FGM peaks. However, sex differences were observed for PM samples, with males demonstrating higher FGM concentrations than females. Sex differences in adrenocortical activity have been observed previously in Carnivora (Monfort et al. 1998; Terio et al. 1999; Wielebnowski et al. 2002), although the differences vary across studies. Results from Monfort et al. (1998) suggested that female African wild dogs had smaller fold increases (~9-fold above basal FGM levels) compared with males (13- and 25-fold increase above basal FGM levels) in response to an ACTH challenge. African wild dog males also had lower pre-ACTH means compared with females (Monfort et al. 1998). Results from Terio et al. (1999) demonstrated higher baseline fecal corticoid concentrations for 1 of 2 male cheetahs compared to the 2 study females. The small sample size used by Terio et al. (1999), however, limits the conclusions that can be drawn about sex differences in cheetahs. In contrast, Wielebnowski et al. (2002) observed greater baseline fecal corticoid concentrations in female North American clouded leopards (*Neofelis nebulosa*) compared to males within a large sample size ($n = 72$). Yet, fold increase of fecal corticosteroid concentrations in response to an ACTH challenge did not differ among sexes. Wielebnowski et al. (2002) suggest that the differences observed may be a result of increased vigilance in females to protect young against infanticide and avoid aggressive encounters with markedly larger males. The potential for reproductive influences on FGMs in our study is unlikely because we observed the study animals during the summer of 2011, which is well outside the coyote breeding season (Carlson and Gese 2008). However, it is possible that sex-related differences in hormone metabolism (Touma and Palme 2005) resulted in the quantitative differences observed by Monfort et al. (1998), Terio et al. (1999), and Wielebnowski et al. (2002). The lack of differences observed in our study may indicate that sex-related differences in coyote metabolism do not exist. Future research looking at metabolic differences among sexes within different seasonal periods (breeding versus nonbreeding) may provide further insight into the patterns observed.

Anthropogenic events documented during this study period allowed us to biologically validate FGM analysis. In both events mentioned above, 4 or 5 of the study animals witnessed a peak promptly following the event (11 h 58 min ± 5 h 53 min and 9 h 21 min ± 5 h 0 min, respectively). However, only 3 individuals (2 males and 1 female) consistently demonstrated increases > 5-fold above the pre-event means for the fan introduction and Pioneer Day. Coincidentally, those 3 individuals were closest to the facility entrance. Examination of our data suggested that proximity to the facility entrance accounts for some of the variance in peak fold values. The most likely explanation for this trend is that coyotes in kennels closer to the entrance have greater visibility of the facility. This result of increased visibility may be 2-fold: the coyote can more readily detect a visitor to the kennel area and also may perceive a decreased sense of cover themselves. Coyotes often use vegetation or brush as cover when frightened (Gehrt 2007), and when unrestricted will move to vantage points to assess threat (Séquin et al. 2003). The perceived lack of adequate cover paired with restricted movement in a kennel environment may provoke stronger responses to human-induced stressors. It is important to note that not all events documented produced pronounced FGM peaks above pre-event means. On 20 July 2011 new coyotes were introduced into the kennel area, presumably a proxy for territorial incursion by novel conspecifics (Gese et al. 1998, 2001) and an acute stressor. However, none of the study animals had fold increases similar in magnitude (> 5-fold) to the other anthropogenic events.

Differences in our FGM results also may reflect individual differences in perception of the event. For instance, FGM concentrations for 1 control female and 1 treatment male were consistently > 5-fold above the pre-event means during the ACTH period, the fan introduction to the kennel, and the Pioneer Day fireworks. Repeated behavioral measures were not noted for this study. However, it is likely that temperament, social status, or both are correlated with the adrenocortical response, as observed in other species. Specifically, results from de Villiers et al. (1997) demonstrated that dominant African wild dogs had higher plasma glucocorticoid levels compared with others. Sands and Creel (2004) provided similar results in gray wolves, because aggressive and dominant individuals had greater basal FGM concentrations. Further, Séquin et al. (2003) demonstrated that coyote response to novelty is closely correlated with social status and temperament, although this study did not directly look at adrenocortical relationships with temperament. Future longitudinal research on temperament, social status, and their relationship to FGMs may provide a unique framework in understanding the variation in responses we have observed here.

To conclude, our results demonstrated robust FGM responses to both the ACTH challenge and 2 anthropogenic events.

The consistency between approximate adrenocortical responses to the ACTH challenge and anthropogenic stimulants suggests that rapid FGM excretion rates in coyotes are species-specific. Heightened metabolism can be an adaptive coping mechanism for persistence in nonnative environments, particularly given that examination of our data suggests that coyotes are responsive to human-associated events. This may have larger implications for coyote management practices in urban and suburban areas. Potential anthropogenic stressors occur relatively frequently in large metropolitan areas. A rapid adrenocortical response to these stressors from resident coyotes may partially enable their success in urban areas. Additionally, these adaptive hormonal responses may be transferred in vitro to developing pups, resulting in a multigenerational mechanism for tolerance to stressors in urban settings. Thus, FGM analysis in the species could prove useful in understanding the proximate mechanisms influencing their overall success as a species. Future work on FGMs could be useful in determining the long-term health of coyote populations and the potential adaptive physiological mechanisms.

Acknowledgments

We thank S. Brummer, J. Schultz, E. Stephenson, and the United States Department of Agriculture–Wildlife Service–NWRC staff for their animal expertise, valuable input into project planning, and time spent caring for the study animals. Likewise, thanks to E. Gese and J. Mateo for their advice at the genesis of this study. Special thanks to D. Armstrong for providing laboratory assistance and help with troubleshooting. Additional thanks to the University of Chicago Hinds Fund and GAANN Fellowship, National Science Foundation, and United States Department of Agriculture–Wildlife Service–NWRC as funding sources for this endeavor.

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Submitted 3 January 2013. Accepted 1 April 2013.

Associate Editor was I. Suzanne Prange.